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An Efficient Method for *in vitro* Clonal Propagation of a Newly Introduced Sweetener Plant (*Stevia rebaudiana* Bertoni.) in Bangladesh

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Abstract: Shootlets were regenerated from nodal explants of *Stevia rebaudiana* Bertoni through axillary shoot proliferation. The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 1.5 mg l^{-1} BA + 0.5 mg l^{-1} Kn. For rooting different concentrations of IBA, NAA and IAA were used and highest rooting percentage (97.66%) was recorded on MS medium with 0.1 mg l^{-1} IAA. The rooted plantlets were hardened and successfully established in soil.

Key words: Stevia rebaudiana Bertoni % node % *in vitro* % sweetner

INTRODUCTION

Stevia rebaudiana Bertoni, is a perennial herb belongs to the Asteraceae family. It is a natural sweetener plant known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and" Honey Leaf", which is estimated to be 300 times sweeter than cane sugar [1, 2]. The leaves of stevia are the source of diterpene glycosides, viz. stevioside and rebaudioside [3]. Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability [4, 5]. Now it is being cultivated in Japan, Taiwan, Philippines, Hawaii, Malaysia and overall South America for food and pharmaceutical products. Products can be added to tea and coffee, cooked or baked goods, processed foods and beverages, fruit juices, tobacco products, pastries, chewing gum and sherbets. In Japan alone, 50 tones of stevioside are used annually with sales valued in order of 220 million Canadian dollars [6]. Also, it has a special importance to diabetic persons and diet conscious. Seeds of stevia show a very low germination percentage [7-10] and vegetative propagation is limited by lower number of individuals [11]. Tissue culture is the only rapid process for the mass propagation of stevia and there have been few reports of *in vitro* growth of stevia [12], *in vitro* micropropagation from shoot tip and leaf [13-18]. Sugarcane Research Institute, Ishwardi, Bangladesh recently introduced this sweetener plant for experimental purpose. The present study was carried out to standardize a suitable protocol for *in vitro* propagation of *S. rebaudiana* Bertoni.

MATERIALS AND METHODS

The twigs (about 5-6 cm) of shoots of pot grown *S. rebaudiana* plants were collected from the Sugarcane Research Institute, Ishwardi. The twigs with node explants were washed in running tap water and then washed again thoroughly by adding a few drops of Tween-20 to remove the superficial dust particles as well as fungal and bacterial spores. They were then surface sterilized with 0.1% mercuric chloride for 5 min followed by rinsing them five times with double distilled water inside the Laminar Air flow chamber. Nodal segments (with a single axillary bud) about 0.5-0.8 cm were prepared aseptically and were implanted vertically on MS medium fortified with specific concentrations of growth regulators (BA, KIN and NAA) singly or in combination adding 30 g l^{-1} sugar (market sugar) and 0.7% Difco Bacto-agar. The pH of the medium was adjusted to 5.7 with 0.1 NaOH before autoclaving at 1.06 kg cm^{-2} and 121°C for 20 min. The cultures were incubated at a constant temperature of 26±1°C with 16 h photoperiod (2000 lux).

Subcultures were done every 21 days interval. Nodal segments from the proliferated shoots were subcultured again for further multiple shoot induction. Regenerated multiple shoots were cut and individual shoots were placed in MS medium containing different concentrations of IBA, NAA and IAA for root induction.

RESULTS AND DISCUSSION

Nodal explants were incubated on MS medium fortified with different concentrations of BA (0.2, 0.5,

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Fig. 1: Axillary shoot proliferation from nodal explants on MS+0.5 mg/l BA +0.2 mg/l NAA after 2 weeks (A), after 4 weeks (B) and after 6 weeks (C) of culture. Adventitious root formation from microcuttings on MS+0.2 mg/l IBA (D). Growth of transplants of *Stevia rebaudiana* on plastic pots (E) and acclimatized on natural environment (F) after 6 months

Table 1: Effects of different concentrations of cytokinin and auxin in MS medium on *in vitro* shoot proliferation from nodal segments of *Stevia*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data (0±SE) were recorded after 6 weeks of culture

Growth regulators (mg lG¹)	% of explant showing shoot proliferation	No. of total shoots per culture	Average length of shoots (cm)
BA 0.2	35.33	2.00±0.45	2.52±0.54
BA 0.5	46.67	3.12±0.17	2.45±0.11
BA 1.0	50.00	3.31±0.30	2.15±0.28
BA 1.5	66.67	5.80±0.24	3.25±0.38
Kn 0.1	40.00	2.10±0.37	2.23±0.11
Kn 0.2	53.33	2.60±0.43	2.32±0.27
Kn 0.5	70.00	3.31±0.30	2.15±0.28
Kn 1.0	62.50	4.40±0.94	2.80±0.93
BA 1.0+Kn 0.5	66.67	4.50±0.20	2.58±0.44
BA 1.0+Kn 1.0	73.33	5.32±0.12	3.45±0.55
BA 1.5+Kn 0.5	85.33	8.75±0.34	4.45±0.45
BA 1.5+Kn 1.0	58.60	3.95±0.03	2.50±0.94
BA 1.0+NAA 0.2	26.00	1.77±0.39	1.27±0.85
BA 1.0+NAA 0.5	35.10	1.82±0.48	1.48±0.39
BA 1.5+NAA 0.2	39.10	2.00±0.12	1.65±0.65
BA 1.5+NAA 0.5	48.25	2.98±0.18	2.00±0.42

Table 2: Effects of IBA, NAA or IAA on adventitious root formation from microcuttings in *S. rebaudiana*. Each treatment consisted of 3 replications and in each replication 10-12 explants were used. Data were recorded after 5 weeks of culture

Type of auxin	Conc.of auxin mg lG¹	% of cuttings rooted	No. of roots per cutting	Average length of the longest root (cm)	Days to mergence of roots	Callus formation at the cutting base
Nil	-	-	-	-	-	-
IAA	0.1	97.66	12.10	2.12	5-12	-
	0.2	92.37	6.35	2.23	6-14	-
	0.5	85.11	4.25	2.88	7-12	-
	1.0	65.38	3.15	3.05	6-15	+
NAA	0.1	70.35	5.55	2.55	7-12	+
	0.2	65.40	7.35	2.38	7-12	+
	0.5	58.20	3.30	2.95	7-12	++
	1.0	51.50	3.10	2.52	7-12	++
IBA	0.1	55.99	2.85	3.01	8-15	-
	0.2	60.72	3.50	3.65	8-12	+
	0.5	45.55	3.65	2.93	8-12	+
	1.0	40.43	3.20	3.55	8-14	++

+, ++, indicate slight, considerable callusing, respectively, -indicates no response

1.0 and 1.5 mg lG¹) and Kn (0.1, 0.2, 0.5 and 1.0 mg lG¹) alone or BA with Kn or with NAA. After six weeks multiple shoots emerged directly from axillary nodes of the cultured explants. The response was best at 1.5 mg lG¹ BA + 0.5 mg lG¹ Kn combination (Table 1) where highest percentage of explants (85.33%) showing shoot proliferation, highest number of total shoots (8.75±0.34) and highest average length of longest shoot (4.45±0.45 cm) were recorded. The response was poor and considerable amount of callus was formed with BA and NAA combination. In the present study 1.5 mg lG¹ BA + 0.5 mg lG¹ Kn was found to be the ideal concentration for high frequency multiple shoot induction. Similar results have already been reported in *Fragaria indica* Andr. [19]. Also this result is in consistent with the findings in papaya [20] as well as in *Eucalyptus grandis* [21].

Microcuttings (longer than 2 cm) taken from the *in vitro* proliferated shoots were implanted on MS medium containing different concentration (0.1, 0.2, 0.5 and 1.0 mg lG¹) of IAA, NAA and IBA for rooting. Maximum root induction (97.66%) was observed in medium fortified with 0.1 mg lG¹ IAA (Table 2). The root induction gradually decreased with increasing concentration of auxin. Except 0.1, 0.2, 0.5 mg lG¹ IAA and 0.1 mg lG¹ IBA callus induction was observed from the cut portion of the shoot. No rooting was obtained on auxin-omitted medium. Proving ideal concentration for root induction; compared to this in *Chrysanthemum morifolium* [22, 23] *Solanum trilobatum* [24], *Carnation* [25], *Pigeon pea* [26], *Vitex negundo* [27], *Peganum harmala* [28] and *Psoralea corylifolia* [29] as well as many other plant species was reported to be the most suitable concentration for root induction.

In vitro rooted shoots were kept under normal growth room conditions for 4 weeks until the induced roots became partially brown. The shoots were then taken out from the growth room, kept under room temperature for 15 days andthen taken out from the culture tubes carefully and the medium attached to the roots was gently washed out with running tap water. The plantlets were transplanted to small polythene bags containing garden soil: compost (2:1) treated with 0.1% Agrason (fungicide). The transplanted plantlets were kept under shade for 15 days and then were transferred to normal environmental conditions. Through this process of acclimatization almost 70% survival was achieved.

By using the method described above, hundreds of clonal plants can be produced from one nodal explant by continuous subculturing of shoot propagules. The multiplication rate that was achieved was not significantly large to be commercially significant, but our results provide a basis for further research in micropropagation of other genotypes of *S. rebaudiana*.

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